IN VITRO MORPHOGENESIS IN PEANUT

BY

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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Peanut, Arachis hypogaea L., improvement by conventional breeding methods has been highly successful, although current limitations to progress are the impetus to alternative genetic manipulation techniques. The purpose of this research was to investigate in vitro morphogenesis in peanut. The areas of study, organogenesis from seed and leaf tissue, anther culture, and somatic embryogenesis, were chosen because of their potential for benefiting improvement programs, namely, for the development of an efficient plant regeneration system, the development of haploid plants for wide hybridizations with normally incompatible, diploid species that carry desirable traits, and for the rapid production of homozygous plants.

The first two areas were designed to assess regeneration of seed and leaf explants. Complete plants were regenerated from cultured embryo axes and embryonated and

deembryonated cotyledons that were whole and sectioned. Multiple shoots arose on 6-benzylaminopurine (BA) supplemented media (0.5-60 mg L^{-1}), with maximum production occurring at 25 mg L^{-1}. Shoot regeneration occurred from the adaxial surface of leaf explants of peanut and perennial peanut, Arachis glabrata Benth., cultured on BA-supplemented media (1-10 mg L^{-1}) with maximum production occurring at 3 and 5 mg L^{-1}. The excised shoots of peanut developed roots upon transfer to medium supplemented with 1 mg 1-napthaleneacetic acid L^{-1}. In vitro-produced plantlets transferred to soil and placed in a greenhouse developed successfully, matured, and set seed. No phenotypic variants were observed among any of the plants produced. Thus, these two systems allow for in vitro production of morphologically normal plants at high frequencies.

The in vitro survival and development of uninucleate microspores over time and the regeneration response of cultured anthers were evaluated. Mitosis and segmentation occurred in 10% of the microspores. Structures resembling embryos emerged from callus originating within anthers. Root organogenesis occurred from somatic tissue-derived callus, however, whole plant regeneration did not occur.

Somatic embryogenesis from the cotyledons of immature zygotic embryos was investigated. Embryogenesis did not occur, and callus was produced from only one of the 1,728 cotyledons cultured.

CHAPTER 1
INTRODUCTION

Arachis hypogaea L., the cultivated peanut, is a crop of major economic importance as a source of protein and oil in both developed and developing countries (Purseglove, 1985). The species is native to Brazil and is grown in tropical and subtropical zones, as well as warm temperate zones. Commercial production is found between 40° N and 40° S latitude (Cobley, 1985). Asia is the largest producer of peanut, followed by Africa, North and Central America, and South America. Among the individual countries, India is the largest producer in the world, followed by China, the United States, Sudan, and Nigeria (Bajaj, 1984).

A number of objectives exist in peanut improvement programs. High and stable yields, drought resistance, high protein and oil content, and earliness of harvest are all characteristics being pursued in various programs (Bajaj, 1984). One of the major objectives, however, is to improve the disease and insect resistance of plants through the production of genetically resistant cultivars (Rugman and Cocking, 1985). Commercial cultivars are susceptible to a range of pests and, consequently, crop yields are reduced (Garren and Jackson, 1973). High levels of resistance to some pests have not been identified in conventional sources of peanut germplasm, and breeders, therefore, have turned their

attention to wild Arachis species which do possess these resistances, among other favorable characteristics (Banks, 1976). For example, Arachis monticola Krap et Rig., Arachis glabrata Benth., and Arachis villosulicarpa Hoehne are resistant to Cercospora sp., while Arachis hagenbeckii Harms and Arachis prostrata Benth. are drought resistant, and Arachis diogoi Hoehne, Arachis marginata Gard., and Arachis villosa Benth. possess high mineral, protein, and oil contents (Bajaj, 1984, Stalker and Wynne, 1979).

The genus Arachis has both diploid (2n=20) and tetraploid (2n=40) species. The cultivated peanut is tetraploid and is not sexually compatible with the wild diploid species. This incompatibility provides a significant barrier to the transfer of known genes for favorable traits from wild species to the cultivated peanut. Numerous interspecific crosses have been attempted to bridge these barriers, but generally have been unsuccessful (Singh et al., 1980, Gregory and Gregory, 1979, Stalker and Wynne, 1979, Johansen and Smith, 1956).

The limitations of conventional breeding methods among Arachis species have enhanced the potential of alternative techniques of genetic manipulation. Plant biotechnology is becoming an important tool for the genetic improvement of crops. Recent advances in genetic manipulation techniques, such as genetic transformation and somatic hybridization, may overcome the barriers to conventional breeding in peanut programs (Bajaj, 1984). Some other in vitro methods that

could be applied to peanut improvement include the production of haploids, the recovery of genetic variability through somaclonal variation, and the in vitro selection of mutants resistant to salt, drought, herbicides, disease, and insects (Bajaj, 1984).

The success of all of these in vitro techniques is dependent upon an efficient system of plant regeneration. Before any meaningful work can be done on the incorporation of these methods into improvement programs, it is essential that basic information on the regeneration response of various peanut tissue explants be obtained (Bajaj, 1984).

Limited research has been reported on the in vitro culture of peanut (Bajaj et al., 1984). However, the literature that does exist for Arachis species indicates a potential for the use of tissue culture techniques for plant regeneration in peanut crop improvement. Complete plantlet regeneration from seedling explants of peanut was reported by Bajaj et al. (1981a) and Mroginski et al. (1981), and from seed explants by Illingworth (1974) and Atreya et al. (1984). Bajaj et al. (1980) reported microspore embryo and callus development from anther cultures of Arachis glabrata Benth., and Martin and Rabechault (1976) obtained anther tissue-derived callus from peanut cultures.

The overall objective of this study was to investigate in vitro morphogenesis in peanut. The four areas of study were organogenesis from seed and leaf tissue, haploid plant production from anther culture, and somatic embryogenesis

from the cotyledons of zygotic embryos. These areas were chosen because of their potential for benefiting peanut improvement programs. The benefits are the development of haploid plants (n=20) for wide hybridizations with normally incompatible, wild, diploid species (2n=20) that carry desirable traits, the rapid production of homozygous plants, the mass propagation of F1 hybrids, haploids, and other rare or desirable plants, and an increase of the available gene pool by utilizing new genetic resources induced in tissue and callus cultures.

The following four chapters deal with research in plant regeneration via organogenesis from seed and leaf explants, microspore development and plant regeneration from anther culture, and somatic embryogenesis from the immature cotyledons of zygotic embryos. The final chapter summarizes the conclusions of the experimental findings of this project and emphasizes areas where additional research may prove productive.

CHAPTER 2
IN VITRO PLANT REGENERATION OF PEANUT FROM SEED EXPLANTS

Peanut, Arachis hypogaea L., is an important crop for the production of oil and protein from the seed in both developed and developing countries. Clonal propagation of favorable lines through the in vitro culture of explants would facilitate breeding and crop improvement programs (Bajaj, 1984). An efficient plant regeneration system is also a prerequisite for genetic transformation studies using Agrobacterium tumefaciens (Smith and Townsend) Conn (Horsch et al., 1985).

Plant regeneration from seed explants has been reported for a number of legumes, including mung bean, Vigna radiata (L.) R. Wilcz., and pigeon pea, Cajanus cajan (L.) Huth (Mathews and Rao, 1984; Mehta and Mohan Ram, 1980). Several reports have described in vitro plant regeneration of peanut from various seed parts. Illingworth (1968, 1974) reported plant regeneration from deembryonated cotyledon sections cultured on basal medium either following or without liquid nitrogen treatment of seeds. The liquid nitrogen caused the cotyledons to separate into fragments. Bhatia et al. (1985) reported development of plants from deembryonated cotyledons and longitudinally sliced cotyledon pieces placed on filter paper moistened with 6-benzylaminopurine (BA) dissolved in

water at 0.05 to 0.5 mg L^{-1}. Atreya et al. (1984) regenerated plants from both excised embryo axes and embryonated cotyledon segments cultured on medium supplemented with 2 mg BA L^{-1}. These studies provide limited quantitative and comparative information on organ yields per explant type, success rates of transplanted in vitro-produced plantlets, and the productivity of various genotypes.

This chapter describes an in vitro regeneration system that maximizes the number of plants attainable from a single seed. The objectives were to (1) compare the regeneration response of five seed explant types, (2) determine the most productive medium for multiple shoot induction, (3) induce root formation and subsequent growth of in vitro-produced plantlets, (4) determine the long-term effects of high cytokinin concentrations during culture stages on mature plant development and function, and (5) assess the response of various genotypes to this tissue culture system.

Materials and Methods

General protocol. Mature peanut seeds were soaked in tap water containing 0.5% antimicrobial P-chloro-M-Xylenol for 2 h. Seed coats were removed, and the seed surface-sterilized in 70% ethyl alcohol for 5 min followed by three separate immersions in 2.6% sodium hypochlorite for 4 min each on a shaker. Seeds were rinsed three times with sterile deionized water. Cotyledons were separated to yield the explant types whole embryonated cotyledon (WEC) and whole deembryonated cotyledon (WDC). Portions of these were

sliced once longitudinally to yield sectional embryonated cotyledon (SEC) and sectional deembryonated cotyledon (SDC) explants. Embryo axes (EA) were excised to yield the fifth explant type. Explants were placed into either 125 X 25 mm glass test tubes containing 20 mL of medium or 100 X 15 mm plastic petri plates containing 25 mL of medium. The nutrient medium consisted of Murashige and Skoog major and minor salts (1962), 3% sucrose, 2 mg glycine L^{-1}, 0.5 mg nicotinic acid L^{-1}, 0.1 mg pyridoxine HCl L^{-1}, and 0.8% Difco agar. Various concentrations of BA (0.05 - 60 mg L^{-1}) and 1-napthaleneacetic acid (NAA) (0 - 1 mg L^{-1}) were added. The pH of the media was adjusted to 5.8 with potassium hydroxide or hydrochloric acid prior to adding the agar. The media were autoclaved at 1.1 kg cm^{-2} for 20 min.

Cultures were maintained at 23°C on a 16/8 h light/dark cycle. General Electric F40CW•RS•WM fluorescent tubes were used which produced approximately 98 μmol m^{-2} s^{-1} when measured 215 mm from the tubes with a Li-190SB (LiCor) quantum sensor (400 - 700 nm range). After 7 d, the cultures with fungal or bacterial contamination were discarded. Contaminant-free cultures were transferred to fresh media at 30-d intervals and routinely examined for morphological development. Unless otherwise stated, all experiments were performed in a completely randomized design.

Various subjects were investigated: (1) explant type; (2) root induction and growth of in vitro-produced plantlets; (3) effect of BA concentration on multiple shoot

production; (4) effects of long-term exposure to BA on shoot production and plant morphology; and, (5) genotype response.

Explant type. The five explant types WEC, WDC, SEC, SDC, and EA of cultivar Florigiant (FG) were cultured on medium supplemented with four concentrations of BA, 0.05, 0.5, 5, and 25 mg L^{-1} for shoot induction and multiplication. This was a 5 X 4 factorial experiment in a completely randomized design, and each treatment combination was replicated 20 times. Cultures were scored at 49 d.

Root induction and growth of in vitro-produced plantlets. Ten shoots from the various treatments were excised and transferred to medium plus 1 mg NAA L^{-1} (Atreya et al., 1984). The shoots were scored for root production at 30 d. Plantlets originating from the five explant types with 5 mm or longer roots were transferred to a soilless mixture (1 peat: 2 perlite: 1 vermiculite) that was autoclaved for 30 min at 1.1 kg cm^{-2}. The plantlets were placed in a greenhouse under 50% shade and 95% humidity for 8 d and 50% shade and 80 to 85% humidity for the following 42 d. The plants were grown to harvest maturity.

Effect of BA concentration on multiple shoot production. Twenty-five WEC explants of FG were placed on each of the media containing eight BA concentration treatments, 0, 10, 20, 25, 30, 40, 50, and 60 mg L^{-1}. After 60 d, the explants were scored for shoot production. Twenty shoots from each of the treatments were excised, placed on rooting medium, and grown to maturity.

Effects of long-term exposure to BA. Whole embryonated cotyledon explants of FG were cultured in 72 160-mL glass jars on both solid and liquid media supplemented with 25 mg BA L^{-1}. The liquid shake cultures contained isolated individual explants grown in 15 mL of medium with agitation. The explants were scored for shoot production at 152, 182, 210, and 238 d after culture initiation. Thirty shoots from both liquid and solid media were excised at these intervals, placed on rooting medium, and scored for root production at 30 d. The plantlets were grown to maturity in the greenhouse.

The percentage of pollen staining was determined as an indication of pollen fertility. Pollen stainability was determined for two anthers of three flowers from each of three plants grown from seed and from three plants that had originated from explants cultured on solid medium supplemented with 25 mg BA L^{-1} for 238 d. The two undehisced anthers from each flower were macerated in a drop of 1% acetocarmine on a slide and viewed after 30 min. The pollen for each slide was classified as stained or unstained in three random samples of 50 grains each.

Genotype response. Whole embryonated cotyledon explants of 20 genotypes were cultured on medium with 25 mg BA L^{-1} for 70 d, and then scored for shoot regenerability. Twenty shoots from each genotype were excised, placed on medium plus 1 mg NAA L^{-1}, and scored for root production at

30 d. The genotypes tested were from a seed collection maintained by the University of Florida (Table 2-1).

Results and Discussion

Explant type. Multiple shoot regeneration occurred from all five explant types on all four BA concentration treatments (Table 2-2). The four cotyledon explant types expanded to approximately twice their original size and turned green within 5 to 7 d after culture initiation. The EA explants and the embryos of explants WEC and SEC began germination within 8 d. Multiple shoots developed from EA, WEC, and SEC explants within 16 d and from WDC and SDC within 35 d.

The deembryonated cotyledon explants, WDC and SDC, regenerated shoots from 22 and 16% of the cultured explants, respectively. These shoots formed from callus that arose at the terminal portion of a protuberance. This protuberance emerged from the base of the cotyledon. These shoots were initially deformed, but eventually developed into normal structures. This feature was previously described by Bhatia et al. (1985). They also reported shoot development from only 12% of deembryonated explants of cultivar TG-17 cultured on medium supplemented with 0.5 mg BA L^{-1}. Illingworth, however, reported shoot regeneration from 85 and 2% of whole and sectional deembryonated cotyledons, respectively, of cultivar Burpee 6212 cultured on basal medium. The variances of the data in these two reports from that

obtained in this study are attributed to BA concentration and genotype effects.

The percentages of explants that produced shoots were greatest among the WEC, SEC, and EA types (Table 2-2). When percentages of responsive explants and numbers of shoots produced per responsive explant were considered together, WEC was found to be superior to the other explant types. Among the four concentrations of BA, the number of shoots formed per responding explant was maximum at the 25 mg L^{-1} level (Table 2-2).

Root induction and growth of in vitro-produced plantlets. When the excised shoots were transferred to the auxin-supplemented medium, roots developed and the resultant plantlets transferred to soil. This ability to root is consistent with the report by Atreya et al. (1984). One or two roots developed from 10% of the shoots that were excised from the various explants within 14 d of transfer to basal medium supplemented with 1 mg NAA L^{-1}. Root growth occurred in 62.5% of all the cultures within 30 d, and approximately 3.5 roots formed per responding shoot. Of 120 plantlets transferred to the greenhouse, 110 (92%) survived the initial 50-d transfer period. Thereafter, 100% of those plantlets surviving developed successfully, matured, and set seed, indicating that any of the five explant types allow shoot production and development of whole plants to maturity. No phenotypic variants were observed in the regenerants.

Effect of BA concentration on multiple shoot formation. Since the largest number of shoots arose from the highest BA concentration tested in the previous experiment, WEC explants of FG were placed on an expanded range of BA concentrations. After 14 d in culture, buds arising from the plumule and cotyledon tissue began to develop into multiple shoots on the 8 BA concentrations tested (Table 2-3). Some of the regenerated shoots (23%) developed from the plumule. The remaining shoots (77%) developed directly from the indented basal region of the cotyledon that surrounds the embryo axis. This tissue, which elongated to within 20 mm and expanded to 10 mm, supported bud formation along its adaxial surface (Fig. 2-1).

Seventy-two percent of the WEC explants formed shoots on the basal medium, which was not significantly different from the frequencies found among the explants cultured on the remaining seven BA concentrations (Table 2-3). The incorporation of BA into the medium therefore was not effective in promoting shoot formation in terms of the number of explants responding. It did, however, have a positive influence on the shoot organogenesis of explants that did respond. The largest number of shoots produced per explant (9.2 - 9.3) was observed on medium supplemented with 25 to 40 mg BA L^{-1} (Table 2-3). Surprisingly, morphologically normal-appearing shoots were produced in relatively large numbers on BA concentrations of up to 60 mg L^{-1}.

Plantlets with leaves and well-developed root systems were obtained 21 to 28 d after placing the excised shoots from all BA concentration treatments on rooting medium. Upon transfer to a greenhouse, mature plants were obtained in 98 d (Fig. 2-2).

Effects of long-term exposure to BA. The levels of BA found to be optimum for shoot production in these experiments were rather high in comparison to the levels used for multiple shoot induction in other plant species (Flick et al., 1983). Therefore, the long-term effects of the 25 mg BA L^{-1} concentration on shoot production and morphogenesis were tested. The use of liquid vs. solid medium for long-term culture also was evaluated. This level of BA (25 mg L^{-1}) allowed increased frequency of shoot formation over time from WEC explants cultured in both liquid and solid media (Table 2-4). At 238 d after culture initiation, a mean of 41.3 and 22.7 shoots had regenerated per responding explant cultured on solid (Fig. 2-3) and liquid (Fig. 2-4) media, respectively.

Solid medium was superior to liquid medium for shoot production both in terms of numbers of shoots produced and because shoots that arose from explants in liquid culture were vitreous. This in vitro physiological anomaly resulted in laminae that were narrow and less chlorophyllous, with outwardly curved margins (Fig. 2-4). All parts of the regenerated shoots exhibited a glassy, translucent appearance.

Shoots were excised at 154, 182, 210, and 238 d after culture initiation, evaluated, and transferred to rooting medium. The number of shoots that developed roots declined with increased culture age for shoots originating on both liquid and solid media. The number of rooted shoots obtained from liquid was, however, much reduced. Approximately 28, 6, 4, and 8%, respectively, of shoots derived from liquid medium produced roots, as compared to 78, 67, 65, and 44% of shoots regenerated on solid medium.

Rooted plantlets from all culture ages transferred to the greenhouse developed and grew to maturity with no morphological variation. The percentages of stainable pollen present in plants originating in vitro after 238 d were not significantly different from those plants grown from seed. Means of 90 and 89% stainability were found among the viewed pollen from in vitro- and seed-produced plants, respectively.

Evaluation of other genotypes. A wide range of peanut genotypes was evaluated for plant production using this regeneration protocol. The range of shooting response among the genotypes was 44 to 94% of all explants cultured (Table 2-5). Varying amounts of shoots regenerated from the responding explants of each genotype. Although there was quantitative variation in both shoot and root development among those genotypes tested, all regenerated plantlets.

In conclusion, these experiments illustrate a procedure for the successful high frequency in vitro regeneration of

plantlets from seed explants of peanut. Optimal quantities of shoots are obtained with WEC explants cultured on solidified medium supplemented with 25 mg BA L^{-1}. Long-term prolific cultures can be produced and maintained, resulting in plants that appear to be normal and fertile.

This procedure can be used for clonal propagation and genetic transformation studies in peanut. In breeding studies of seed nutritional composition, these procedures would allow for the production of multiple plants from one cotyledon, while the other cotyledon is utilized for chemical analyses.

Table 2-1. Peanut genotypes used for seed explant cultures.

Genotype†	Market type§	Plant growth habit¶
Florunner cv.	Runner	Runner
Sunrunner cv.	Runner	Runner
Chico cv.	Spanish	Bunch
NC-7 cv.	Virginia	Spreading bunch
Florigiant cv.	Virginia	Runner
392-x ‡	Virginia	Runner
393-7	Virginia	Runner
487B	Virginia	Spreading bunch
435-OL-2	Spanish	Bunch
562A	Spanish	Bunch
803-1	Runner	Runner
623B	Valencia	Bunch
640A	Valencia	Bunch
558A	Spanish	Bunch

† cv. = cultivar.

‡ Component lines of Florigiant.

§ Virginia - alternate branched form with large seeds (1100 seed kg^{-1}; Runner - alternate branched form with small seeds (1540 seed kg^{-1}); Spanish - sequential branched form with 2-seeded pods; Valencia - sequential branched form with 3- to 4-seeded pods.

¶ Runner - prostrate growth habit; Bunch - upright growth habit occurring in sequential branched forms; Spreading bunch - upright growth habit occurring in alternate branched forms.

Table 2-2. In vitro production of Florigiant peanut shoots, as influenced by 6-benzylaminopurine (BA) level and explant type.

BA Level mg L^{-1}	No. (%) responsive explants†					Mean no. shoots per responding explant‡				
	WEC§	WDC	SEC	SDC	EA	WEC	WDC	SEC	SDC	EA
0.05	10(90)	3(16)	10(66)	2(11)	19(95)	2.1	1.0	1.7	1.0	2.2
0.50	8(66)	2(10)	10(59)	5(25)	14(93)	2.5	1.5	1.2	1.0	2.2
5.00	10(90)	4(20)	9(69)	2(12)	13(86)	3.0	2.8	2.4	4.0	2.5
25.00	17(94)	8(40)	15(79)	3(15)	20(100)	12.2	12.0	4.0	3.7	3.1

† The response of explants is based on explant type and BA level as dependent treatments at 1% level, $X^2 = 31.71$.

‡ The least significant difference test values for comparing two BA levels for any explant type are 4.17 at the 5% level of significance and 5.47 at the 1% level.

§ WEC = whole embryonated cotyledon, WDC = whole deembryonated cotyledon, SEC = sectional embryonated cotyledon, SDC = sectional deembryonated cotyledon, EA = embryo axis.

Table 2-3. Shoot production from whole embryonated cotyledon explants of Florigiant peanut on varying levels of 6-benzylaminopurine (BA).

BA concentration (mg L^{-1})	No. (%) responding explants†	Mean no. of shoots per responding explant	Difference from control
0 (Control)	18 (72)	1.4	--
10	15 (68)	4.4	3.0 ns
20	14 (61)	8.2	6.8 **
25	15 (71)	9.3	7.9 **
30	16 (73)	9.2	7.8 **
40	14 (61)	9.2	7.8 **
50	13 (62)	6.6	5.2 **
60	14 (61)	7.9	6.5 **

**Significant at the 0.01 probability level. ns = not significant.

† No significant differences among treatments at 5% level, $X^2 = 3.09$.

Table 2-4. Comparison of mean shoot production from 36 whole embryonated cotyledon explants of Florigiant peanut on solid and liquid media, supplemented with 25 mg 6-benzylaminopurine (BA) L^{-1}, over time.

	Days after culture initiation			
Media	154	182	210	238
Solid	11.5	16.8	31.5	41.3
Liquid	7.7	10.4	16.8	22.7
Difference	3.8***	6.3***	14.7***	18.5***

*** Significant at the 0.001 probability level.

Table 2-5. Morphogenic responses of peanut cultivars in culture.

Cultivar	Shoot Production†		Root Production‡	
	No. (%) of Responding Explants	No. per Responding Explant§	No. (%) of Responding Shoots	No. per Responding Shoot§
Florunner	7 (44)	13.1ab	17 (85)	6.4b-e
Sunrunner	9 (50)	10.1a-d	12 (60)	7.1bcd
Florigiant	7 (70)	14.4a	12 (60)	4.5def
Chico	12 (75)	6.6de	5 (25)	1.4f
NC-7	9 (75)	7.6cde	13 (65)	7.5bcd
487-B	10 (71)	6.1de	10 (56)	4.6def
435-OL-2	7 (58)	4.6e	13 (68)	6.2b-e
393-7	11 (69)	9.3b-e	8 (42)	3.1ef
392-B	16 (84)	6.1de	17 (85)	11.3a
392-C	14 (78)	6.7de	16 (80)	8.9abc
392-E	11 (65)	7.2cde	15 (75)	9.2ab
392-F	15 (79)	5.7de	13 (65)	11.1a
392-G	14 (82)	6.1de	12 (60)	7.3bcd
392-H	9 (56)	4.6e	11 (55)	6.5b-e
392-I	11 (73)	5.9de	13 (65)	7.1bcd
562A	16 (94)	12.1abc	18 (90)	4.3def
803-1	15 (88)	6.1de	13 (65)	7.0bcd
623B	17 (94)	8.7b-e	17 (85)	3.9def
640A	7 (50)	10.3a-d	19 (95)	7.5bcd
558A	11 (79)	7.9cde	18 (90)	5.3cde

† Production of shoots from whole embryonated cotyledon explants cultured on 25 mg 6-benzylaminopurine L^{-1} for 10 weeks.

‡ Production of roots from excised shoots placed on 1 mg 1-napthaleneacetic acid L^{-1} for 30 days.

§ Mean separation by Duncan's multiple range test at 5% level.

Figure 2-1. Multiple shoot production from the adaxial surface of a whole embryonated cotyledon explant of Florigiant peanut.

Figure 2-2. Plants of Florigiant peanut regenerated from whole embryonated cotyledon explants cultured in vitro on media supplemented with 0, 10, 20, 25, 30, 40, 50, and 60 mg 6-benzylaminopurine L^{-1}.

Figure 2-3. Multiple shoot production from a whole embryonated cotyledon explant of Florigiant peanut cultured on solid medium supplemented with 25 mg 6-benzylaminopurine L^{-1}.

Figure 2-4. Multiple shoot production from a whole embryonated cotyledon explant of Florigiant peanut cultured in liquid medium supplemented with 25 mg 6-benzylaminopurine L^{-1}.

CHAPTER 3
ORGANOGENESIS FROM CULTURED LEAF TISSUE OF PEANUT AND PERENNIAL PEANUT

Organogenesis is the process of regenerating plants in vitro from callus or differentiated tissue. This process involves the culturing of explant tissue on a defined medium containing an appropriate hormone and nutrient regime under a controlled set of environmental conditions. The cells grown in culture divide and differentiate to form shoots and/or roots. Shoots which form without roots generally are separated and placed on a medium favoring the development of roots.

In vitro regeneration allows for the potential production of many plants from a single plant exhibiting or carrying traits desirable for improved cultivars. It also provides an alternative vegetative propagation means for crop plants that are normally not seed propagated.

One such species is the perennial peanut, Arachis glabrata Benth., which is a tropical rhizomatous legume that produces high quality forage that can be grazed, hayed, or made into silage (Prine et al., 1981). It is a new crop to southeastern U.S. farmers, and establishment costs are high (Williams, 1988). The ability to initiate fields via established transplant material from in vitro-produced whole plants may greatly reduce the time and costs incurred.

A large number of plant species have been regenerated from in vitro culture (Binding and Schroeren, 1984), although some of the more important crops, notably legumes, have lagged behind in development of regeneration techniques. Flick et al. (1983) provides a list of 25 legume species which have been regenerated in vitro, although in most cases regeneration occurred at low frequencies. Little work has been reported on peanut, Arachis hypogaea L., but the accumulated literature from the last seven years demonstrates the potential of in vitro techniques for its improvement.

Mroginski et al. (1981) induced shoot regeneration from the callus of immature leaves of peanut cv. Colorado Manfredi cultured on medium supplemented with 1 mg L^{-1} each of 6-benzylaminopurine (BA) and 1-napthaleneacetic acid (NAA), and Pittman et al. (1983) later extended this study to 28 genotypes. All genotypes produced callus, 78% produced shoots, and 19% produced roots. Callus production generally started on the basal end of the leaflet. Shoot meristems developed on the periphery of the callus. Histological examination of the cultures revealed that embryos and shoot meristems originated from adaxial epidermal cells near the midrib of the leaflet.

Pittman et al. (1984) and Johnson and Pittman (1986) later studied the various factors that affected the in vitro performance of leaflet explants of Arachis villosulicarpa Hoehne, a species that carries a high level of resistance to

Cercospora arachidicola Hori and Cercosporidium personatum (Berk. and Curt.) Deighton. They found that explant orientation was important, with twice as many explants forming shoots when the abaxial epidermis, rather than the adaxial, was in contact with the agar. They also found that the presence of midrib in the explants reduced the number of shoots formed per culture, and larger numbers of shoots could be obtained by increasing the light intensity to 53 μmol m^{-2} s^{-1}.

Bajaj et al. (1981a) established callus cultures from leaf explants of cultivated peanut cultured on medium supplemented with 2 mg indole-3-acetic acid (IAA) L^{-1} and 0.5 mg kinetin (KN) L^{-1}. Sukumar and Rangasamy (1984) studied the callus morphology and organogenic potential of leaf explants from seven Arachis species and found these explants to be variable in response. The calli differed in growth rate and texture, being either smooth or nodular and firm or soft. Three species developed roots and no shoots formed in the calli of any species. Narasimhulu and Reddy (1983) reported sporadic shoot development from leaf callus cultured on medium supplemented with 1 mg BA L^{-1} and 0.4 mg NAA L^{-1}.

The above studies provide information on the regenerative potential of leaf tissue of Arachis species cultured on low levels of cytokinin. The studies report shoot organogenesis, but provide limited quantitative information on organ yields and their subsequent development. This chapter

investigates the organogenic capacity of peanut leaf tissue cultured on medium supplemented with elevated concentrations of cytokinin. It also investigates the regeneration response of 20 peanut cultivars and two perennial peanut cultivars.

Materials and Methods

Peanut cv. Florigiant (FG) seeds were surface sterilized in 70% ethyl alcohol for 5 min followed by three separate immersions in 2.6% sodium hypochlorite for 4 min each on a shaker. Seeds were rinsed three times with sterile deionized water. The seeds were germinated at 23°C on a 16/8 h light/dark cycle on 0.8% Difco agar in water in 100 x 15 mm plastic petri plates. After 8 d, 5-mm leaflets were excised and placed in petri plates containing 25 mL of medium. The nutrient medium consisted of Murashige and Skoog major and minor salts (1962), 3% sucrose, 2 mg glycine L^{-1}, 0.5 mg nicotinic acid L^{-1}, 0.1 mg pyridoxine hydrochloride L^{-1}, 1 mg NAA L^{-1}, and 0.8% Difco agar. A range of BA concentrations, 1, 3, 5, and 10 mg L^{-1}, was tested. Each of these four treatments was replicated 45 times in a completely randomized design. The pH of the media were adjusted to 5.8 with potassium hydroxide or hydrochloric acid prior to adding the agar. The media were autoclaved at 1.1 kg cm^{-2} for 20 min.

Cultures were maintained at 23°C with a 16 h daylength. General Electric F40CW•RS•WM fluorescent tubes were used which produced approximately 98 μmol m^{-2} s^{-1} when measured

215 mm from the tubes with a Li-190SB (LiCor) quantum sensor (400 - 700 nm range). After 10 d, the cultures with fungal or bacterial contamination were discarded. Contaminant-free cultures were transferred to fresh media at 30-d intervals and routinely examined for morphological development. When buds arose they were isolated and transferred to 125 x 25 mm glass test tubes, containing 20 mL of nutrient medium supplemented with only 5 mg BA L^{-1} to promote shoot elongation. When shoots reached 15 mm they were transferred to the initial culture medium containing 1 mg NAA L^{-1} to initiate roots (Atreya et al., 1984). Plantlets were transferred to a 1 peat: 2 perlite: 1 vermiculite greenhouse potting medium that was autoclaved for 30 min at 1.1 kg cm^{-2}. The plantlets were placed in a greenhouse under 50% shade and high humidity for 40 d and then grown to maturity.

Genotype response. Thirty-five leaf explants from 20 peanut genotypes were cultured on nutrient medium supplemented with 5 mg BA L^{-1} for 90 d, and then scored for shoot and root development. The genotypes tested were from a seed collection maintained by the University of Florida (Table 3-1).

Perennial peanut. Leaf explants from two perennial peanut cultivars, 'Florigraze' and 'Arbrook', were cultured on media supplemented with 1 mg NAA L^{-1} plus the BA concentration treatments 1, 3, 5, and 10 mg L^{-1}. Leaflets, 5 to 10 mm long, were excised from mature plants and sliced along the midribs into two explants. The explants were surface

sterilized in 70% ethyl alcohol for 5 min and in 2.6% sodium hypochlorite for 10 min. Explants were rinsed three times with sterile deionized water. Sixty explants from each cultivar were cultured on each BA concentration treatment, and evaluated 90 d after culture initiation.

Results and Discussion

Within six days of culture initiation, FG leaflets began to expand and thicken with all media treatments. Callus production and organogenesis occurred on all four BA concentration treatments (Table 3-2). Callus developed from the adaxial surface within 21 d, while bud tissue developed from the adaxial surface 36 d after culture initiation (Fig. 3-1). Roots developed from the callus within 45 d.

Ninety-six percent of the explants developed callus on the BA 1 mg L^{-1} treatment, which was not significantly different from the frequencies found among the leaflets cultured on the remaining three treatment levels. The concentration of BA did, however, have a significant influence on the capacity of explants to produce organs. The largest number of explants that developed bud tissue (38%) was observed on medium supplemented with 5 mg BA L^{-1}. This number is much lower than that obtained by Mroginski et al. (1981), who reported 92% bud tissue development from cultured leaf explants of peanut cv. Colorado Manfredi. Thirty-five percent of the explants cultured on 1 mg BA L^{-1} produced roots (Table 3-2). As the BA concentration increased, the number of explants that produced roots

declined, with no roots formed at a concentration of 10 mg L^{-1}. These results are comparable to the rooting results reported by Mroginski et al. (1981). The formation of both bud tissue and roots from a single explant was not observed.

Isolated bud tissue was then transferred to basal medium supplemented with 5 mg BA L^{-1} to promote shoot differentiation and elongation, and 84% percent of these cultures developed one to three shoots (Fig. 3-2) within 120 d of transfer. When these shoots were transferred to the auxin-supplemented medium, 100% formed plantlets with one or two roots within 35 d, and were transferred to soil. The capacity of shoots regenerated from peanut leaf tissue to root upon transfer to medium supplemented with 1 mg NAA L^{-1} was previously described by Narasimhulu and Reddy (1983). Of 27 plantlets transferred to the greenhouse, 23 (85%) survived the initial 40-d transfer period and developed successfully to maturity. No phenotypic variants were observed in the regenerants.

Evaluation of other genotypes. A wide range of peanut genotypes was evaluated for organogenic responsiveness. Although all genotypes produced callus in similar frequencies, significant differences in bud and root formation occurred among those tested (Table 3-3). The majority of cultivars developed bud tissue during the 90-day culture period. Such variation in response among genotypes was also reported by Pittman et al. (1983), who evaluated 28

genotypes. Much variation in callus production and organogenesis was noted.

Perennial peanut. Within 30 d of culture initiation, 95% of the leaf explants formed callus. The number of explants of Arbrook and Florigraze which formed callus was not significantly different among the four treatments. By 50 d, dense friable callus containing many shoot meristems developed from the explants (Fig. 3-3). The concentration of BA had a positive influence on the number of calli yielding meristems (Table 3-4). The largest number of cultures forming meristems was observed on media supplemented with 3 and 5 mg BA L^{-1}. A small quantity of these meristems (10%) continued growth and differentiation towards shoot development (Fig. 3-4) upon transfer to basal medium supplemented with 5 mg BA L^{-1}, although additional studies are required to continue this protocol for complete plant regeneration.

Table 3-1. Peanut genotypes used for leaflet cultures.

Genotype†	Market type§	Plant growth habit¶
Florunner cv.	Runner	Runner
Sunrunner cv.	Runner	Runner
Chico cv.	Spanish	Bunch
NC-7 cv.	Virginia	Spreading bunch
Florigiant cv.	Virginia	Runner
392-x ‡	Virginia	Runner
393-7	Virginia	Runner
487B	Virginia	Spreading bunch
435-OL-2	Spanish	Bunch
562A	Spanish	Bunch
803-1	Runner	Runner
623B	Valencia	Bunch
640A	Valencia	Bunch
558A	Spanish	Bunch

† cv. = cultivar.

‡ Component lines of Florigiant.

§ Virginia - alternate branched form with large seeds (1100 seed kg^{-1}; Runner - alternate branched form with small seeds (1540 seed kg^{-1}); Spanish - sequential branched form with 2-seeded pods; Valencia - sequential branched form with 3- to 4-seeded pods.

¶ Runner - prostrate growth habit; Bunch - upright growth habit occurring in sequential branched forms; Spreading bunch - upright growth habit occurring in alternate branched forms.

Table 3-2. Morphogenic response of Florigiant peanut leaf explants to varying concentrations of 6-benzyl-aminopurine (BA).

BA Concentration (mg L^{-1})	No. (%) responding explants		
	Callus Development†	Bud Formation‡	Root Formation§
1	43(96)	2(4)	16(35)
3	44(98)	4(9)	11(25)
5	43(96)	17(38)	3(7)
10	42(94)	9(20)	0(0)

† NS = No significant differences at the 0.05 probability level.

‡ The least significant difference (LSD) test values for comparing two BA concentrations are 21 at the 5% level of significance and 28 at the 1% level.

§ The LSD test values for comparing two BA concentrations are 20 at the 5% level of significance and 26 at the 1% level.

Table 3-3. Morphogenic response of leaf explants of peanut cultivars in culture.

Cultivar	No (%) responding explants†		
	Callus Development‡	Bud Formation§	Root Formation¶
Chico	30(93)	7(23)	0
Florigiant	33(100)	5(15)	2(7)
Florunner	33(98)	7(20)	3(10)
Sunrunner	30(100)	6(20)	4(13)
562A	35(100)	0	0
558A	32(96)	9(27)	0
623B	31(97)	4(14)	0
640A	34(96)	2(5)	0
803	30(94)	6(19)	1(4)
487B	33(93)	0	0
393-7	34(96)	0	0
435-OL-2	35(100)	8(22)	0
NC-7	34(100)	4(12)	0
392-B	31(92)	2(5)	4(12)
392-C	33(93)	2(6)	2(6)
392-E	31(92)	9(26)	0
392-F	30(94)	7(22)	0
392-G	33(93)	4(10)	0
392-H	35(100)	3(9)	2(6)
392-I	35(100)	5(15)	4(11)

† Leaf explants were cultured on medium supplemented with 5 mg 6-benzylaminopurine L^{-1} for 90 days.

‡ NS = No significant differences at the 0.05 probability level.

§ Significant at the 0.005 probability level, $x^2 = 44.89$.

¶ Significant at the 0.005 probability level, $x^2 = 42.87$.

Table 3-4. Morphogenic response of leaf explants from perennial peanut cultivars Arbrook and Florigraze to varying concentrations of 6-benzylaminopurine (BA).

BA Concentration ($mg\ L^{-1}$)	No. (%) calli with shoot meristems†	
	Arbrook‡	Florigraze‡
1	15(27)	10(17)
3	32(59)	26(47)
5	30(57)	24(41)
10	19(36)	17(32)

† Percent response was determined 90 days after culture initiation.

‡ The least significant difference test values for comparing two BA concentrations for each cultivar are 23 at the 5% level of significance and 30 at the 1%.

Figure 3-1. Bud tissue originating from the adaxial surface of a leaf explant of Florigiant peanut cultured on medium supplemented with 1 mg 1-napthaleneacetic acid L^{-1} and 3 mg 6-benzylaminopurine L^{-1}.

Figure 3-2. Shoot differentiation from bud tissue of Florigiant peanut cultured on medium supplemented with 5 mg 6-benzylaminopurine L^{-1}.

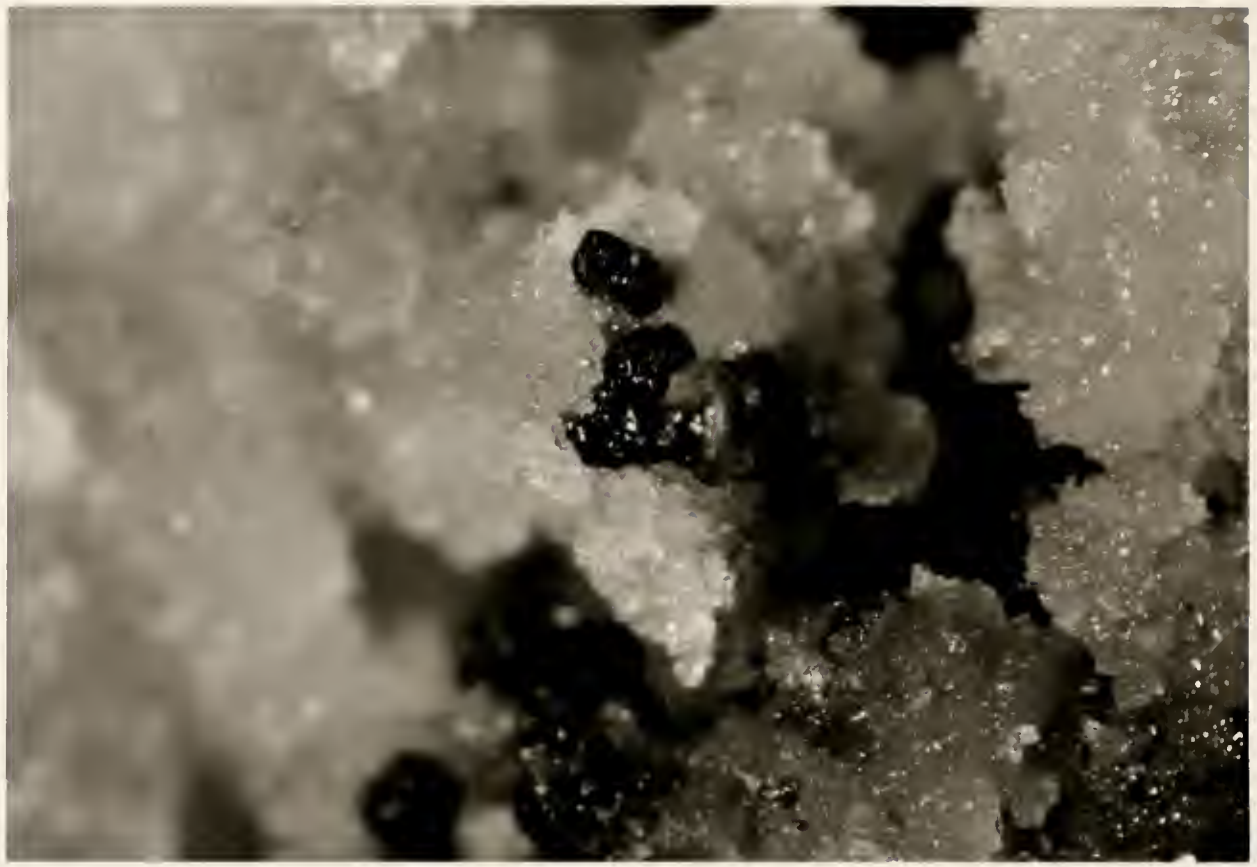

Figure 3-3. Organogenic leaf callus of Arbrook perennial peanut cultured on medium supplemented with 1 mg 1-napthaleneacetic acid L^{-1} and 3 mg 6-benzyl-aminopurine L^{-1}.

Figure 3-4. Shoot differentiation from leaf callus of Arbrook perennial peanut cultured on medium supplemented with 5 mg 6-benzylaminopurine L^{-1}.

CHAPTER 4
IN VITRO CULTURE OF PEANUT ANTHERS

The tissue culture of anthers is a means of obtaining haploid plants, which are plants possessing the gametophytic number of chromosomes in their sporophytes, from microspores. The technique involves plating anthers at the proper developmental stage on a defined medium. Uninucleate microspores before or during mitosis are most inductive towards embryo development (Bajaj, 1983). The anthers are dissected out of unopened, sterilized flower buds and plated directly onto an appropriate agar medium. The medium and procedures are adjusted so that the microspores are induced to divide. Their division results in either direct development into embryos through stages analogous to those of normal diploid seed embryos or the production of an undifferentiated callus, which when transferred to a regeneration medium will differentiate shoots and roots (Reinert and Bajaj, 1977).

Plants regenerated from anther culture will often have variation in ploidy levels. Nonhaploid plants can conceivably arise from 2n microspores, polyploidization of pollen callus cells, or from callus derived from somatic tissue (Sink and Padmanabhan, 1977). The direct development of embryos from uninucleate microspores theoretically insures haploidy and is therefore preferred to the callus pathway.

Guha and Maheshwari (1964) first reported haploid plant production from the pollen of Datura inoxia Mill., and since that time anther culture has been tried with a number of economic species (Bajaj, 1983). This technique is valuable because haploid plant development offers a rapid means for the production of homozygous plants. They also can facilitate the detection of useful traits, unique recombinants, and mutations because they possess only one allele at each locus. They also may allow for wide hybridizations with species having similar chromosome numbers (Bajaj, 1984). With Arachis sp. anther culture progress has been limited to the production of nonhaploid plants (Bajaj et al., 1981b, Mroginski and Fernandez, 1980, and Seitz et al., 1985) and to the production of multicellular microspores, an early stage of embryogenesis (Bajaj et al., 1980, 1981b and Mroginski and Fernandez, 1979).

Bajaj et al. (1980, 1981b) reported the induction of pollen embryogenesis in anther cultures of Arachis villosa Benth. and Arachis glabrata Benth. In the first study they cultured anthers of A. glabrata and peanut, Arachis hypogaea L., at the uninucleate and binucleate microspore stages on Murashige and Skoog medium supplemented with 4 mg indole-3-acetic acid (IAA) L^{-1} and 2 mg kinetin (KN) L^{-1}. The microspores of A. glabrata divided to form embryos and, in some cases, proliferated to form callus. The progress of the peanut microspores was not reported. In the later report they cultured anthers of A. villosa and peanut cv. M 13 on

the same medium when microspores were undergoing mitosis. Callus developed within 21 d from 11% and 13% of the anthers, respectively, and only the microspores of A. villosa were reported to have undergone repeated nuclear and cell divisions to form embryos. These microspores proliferated to form callus which was more compact than callus obtained from anther tissue. Within 4 wk, the two callus types could not be distinguished from each other. The calli were transferred to medium supplemented with 1 mg 1-naphthaleneacetic acid (NAA) L^{-1} and 2 mg 6-benzylaminopurine (BA) L^{-1} and began to form organs within 7 d. Eighteen percent of the calli from M 13 produced roots with occasional shoot formation, and 55% of the calli from A. villosa differentiated shoots which were transferred to medium supplemented with 0.5 mg NAA L^{-1} for rooting. It was not reported whether these organs differentiated from microspore-derived callus or from anther tissue-derived callus. The chromosome numbers of M 13 callus cells showed a range of variation from 20 to 80, while the chromosome numbers of A. villosa callus cells were not reported.

Mroginski and Fernandez (1979) cultured anthers of peanut cv. Colorado Manfredi, Arachis correntina (Burk) Krap. et Greg., and A. villosa on medium supplemented with 2 mg NAA L^{-1} and 0.5 mg BA L^{-1}. Callus was produced from 60%, 58%, and 40% of the anthers, respectively. The microspores of Colorado Manfredi degenerated within 4 d of culture, and callus developed from somatic tissue within 21 d. On the

other hand, anthers of A. correntina and A. villosa, both at 2n=20, yielded callus cells with 10 chromosomes. Multicellular microspores were also found. These workers (1980) cultured anthers of Arachis lignosa (Chod. et Hassl.) Krap. et Greg. at the uninucleate microspore stage on the same medium, and approximately 56% of the anthers developed callus from somatic tissue within 30 d. Plantlets developed on 78% of these calli when transferred to medium with 0.01 mg BA L^{-1}.

Martin and Rabechault (1976) cultured stamens of peanut on medium supplemented with 0.46 mg 2,4-dichlorophenoxyacetic acid (2,4-D) L^{-1}, 0.4 mg NAA L^{-1}, and 0.45 mg KN L^{-1}. Microspore division was arrested as soon as the stamens were cultured. These anthers produced callus at the base of the filament within 15 d. Pittman (1981) cultured anthers containing microspores at the late tetrad stage of development from 10 genotypes representing seven Arachis species. They were cultured on solid medium supplemented with 1.86 mg NAA L^{-1} and 0.02 mg KN L^{-1}, and in liquid medium with 2 mg NAA L^{-1} and 0.5 mg BA L^{-1} as float cultures. The anthers produced callus in both cultures at the site where the filaments were removed.

Seitz et al. (1985) isolated a highly morphogenic callus line from the anthers of Arachis paraguariensis Benth. cultured in both solid and liquid media supplemented with 0.2 mg 4-amino-3,5,6-trichloropicolinic acid (picloram) L^{-1} and 0.5 mg BA L^{-1}. This callus developed from filament

remnants in both systems and supported shoot development within 8 to 10 wk of culture initiation. These shoots regenerated roots upon transfer to medium supplemented with 4 mg NAA L^{-1}.

In conclusion, success with anther culture in peanut has been limited. This limited success may be due to genotype and/or media constraints. Haploid callus cells of M 13 peanut were found in the anther cultures of one study (Bajaj et al., 1981b), however, in another study the microspores of Colorado Manfredi were found to degenerate in 4 d (Mroginski and Fernandez, 1979). Both studies used media which successfully induced microspore division in the species A. villosa. Therefore, genotype may play a greater role in microspore division induction than medium components. Embryogenesis was induced in cultures of two diploids, A. villosa and A. correntina, (Bajaj et al., 1981b, Mroginski and Fernandez, 1979) and in one tetraploid, A. glabrata (Bajaj et al., 1980). In each case the microspores underwent repeated divisions resulting in the formation of both multicellular microspores and callus.

This chapter investigated the culture of anthers from three peanut cultivars. The cultures were evaluated for the formation of embryos and/or callus from microspores, and subsequent organ differentiation. The media used in the studies by Bajaj et al. (1981b) and Mroginski and Fernandez (1979) were evaluated for their efficacy. The objectives of the chapter were to (1) stage anthers at the uninucleate

microspore stage to determine the proper flower bud sizes for culture, (2) follow the survival and developmental course of microspores over time, and (3) assess the morphogenic response of the three cultivars.

Materials and Methods

Flower buds of peanut cv. Florigiant (FG), Florunner (FR), and Chico (CH) were staged to establish a relationship between microsporogenesis and bud length. Flower buds of various sizes from four greenhouse-grown plants of each cultivar were collected before 0800 h. Bud length was determined, using a dissecting microscope, from the base to the tip of the sepals. Four oblong anthers from each of three buds of the same size were evaluated. A total of 96, 84, and 60 anthers from FG, FR, and CH, respectively, were studied. The four oblong anthers from each bud were macerated individually in a drop of 1% acetocarmine on a slide. Sixty microspores from each anther were viewed under 200X magnification after 30 min and classified according to microspore development stage.

Microspore development. The survival and development of microspores over time was followed in FG by viewing the microspores at 7-d intervals from anthers cultured on an embryo induction medium. Oblong anthers containing uninucleate microspores were surface sterilized in 70% ethyl alcohol for 1 min and in 2.6% sodium hypochlorite for 5 min. They were rinsed two times with sterile deionized water. Sixty anthers were placed in petri plates containing 25 mL

of medium. The nutrient medium consisted of Murashige and Skoog's major and minor salts (1962), 3% sucrose, 2 mg glycine L^{-1}, 0.5 mg nicotinic acid L^{-1}, 0.1 mg pyridoxine HCl L^{-1}, and 0.8% Difco agar. It was supplemented with 4 mg IAA L^{-1} and 2 mg KN L^{-1} (Bajaj et al., 1981b). The pH of the medium was adjusted to 5.8 with potassium hydroxide prior to adding the agar. The medium was autoclaved at 1.1 kg cm^{-2} for 20 min. Cultures were maintained at 23°C on a 16/8 h light/dark cycle. General Electric F40CW•RS•WM fluorescent tubes were used which produced approximately 90 μmol m^{-2} s^{-1} when measured 215 mm from the tubes with a Li-190SB (LiCor) quantum sensor (400 - 700 nm range). Microspore observations were made 4 d after culture initiation and thereafter at 7-d intervals for 70 d. Three anthers were removed from culture, macerated in 1% acetocarmine, and viewed individually for microspore development.

Morphogenic response. Oblong anthers containing uninucleate microspores of FG, FR, and CH were surface sterilized and cultured on the agar solidified nutrient medium supplemented with either 2 mg NAA L^{-1} and 0.5 mg BA L^{-1} (Medium M) as used by Mroginski and Fernandez (1979), or 4 mg IAA L^{-1} and 2 mg KN L^{-1} (Medium B) as used by Bajaj et al. (1981b). After 7 d, the cultures with fungal or bacterial contamination were discarded. Cultures were transferred to fresh media at 35 d and routinely examined for morphological development. Cultures that developed callus within 35 d of culture initiation were transferred to media supplemented

with either 0.01 mg BA L^{-1} (Mroginski and Fernandez, 1979), or 1 mg NAA L^{-1} and 2 mg BA L^{-1} (Bajaj et al., 1981b), respectively, to initiate organogenesis. Ninety anthers of each cultivar were cultured in a completely randomized design.

Results and Discussion

Four oblong anthers from each of three flower buds of the same size were stained in acetocarmine to assess the stage of development of the microspores. Microspore development was synchronous; the 12 anther samples of each bud size of FG, FR, and CH consistently contained 97 to 100% of the total number of microspores at the same stage of development. Table 4-1 presents the relationship between the flower bud lengths and the stage of development of the microspores for the three cultivars. Buds, 1.5 to 2 mm, 2 to 3.5 mm, and 1 to 1.5 mm long, of FG, FR, and CH, respectively, contained microspores which were uninucleate. Current knowledge indicates that microspores at the uninucleate stage are most inductive towards embryogenesis (Bajaj, 1983). Anthers cultured in the following experiments were obtained from flower buds of these sizes.

Microspore development. Acetocarmine preparations of FG anthers showed that 4 d after culture initiation the majority of microspores remained unchanged. Comparatively, Mroginski and Fernandez (1979) reported that 100% of the microspores from peanut cv. Colorado Manfredi degenerated within 4 d of culture initiation. At 7 d 25% of the FG

microspores were shriveled, and the remaining were lightly stained uninucleate (Fig. 4-1). At 28 d 50% had degenerated, 20% were uninucleate, and 30% were binucleate (Table 4-2). An interesting aspect of the binucleate microspores was a differential response to staining by acetocarmine. In one group the cytoplasm stained darkly, partially obscuring the two nuclei (Fig. 4-2). In the other group the cytoplasm stained lightly, indicating a lower starch content and allowing the microspore segmentation and nuclei to be easily viewed (Fig. 4-3). This double response phenomenon has been reported in tobacco by Sunderland and Wicks (1969). They found that the densely staining type developed subsequently like normal pollen and that the lightly staining type proved to be embryoidal. They observed both binucleate microspore types 2 d after culture initiation. Mitosis occurred in the embryoidal type on the 6th d of culture, and multicellular embryos were observed on the 10th d.

Further development of both the lightly and densely staining binucleate microspores of FG did not occur during the 70-d observation period. Similar quantities of degenerated, uninucleate, densely staining binucleate, and lightly staining binucleate microspores were observed at each of the remaining six observations (Table 4-2).

Morphogenic response. The excised anthers of FG, FR, and CH cultured at the uninucleate microspore stage on M and B media initiated callus within 13, 20, and 20 d, respectively, and callus masses were formed within 35 d. In most

cases, callus first appeared at either the cut end of the stamen filament or on the anther wall. A higher percentage of responding anthers cultured on Medium M than on B for each cultivar was noted (Table 4-3). When callus was transferred to the organ differentiation media, roots began to differentiate in FG cultures within 11 d. The formation of roots provides evidence for morphogenesis and possible plantlet regeneration from somatic anther tissue of FG. Organogenesis from the callus of peanut anthers was first reported by Bajaj et al. (1981b), who observed root development in 18% of cv. M 13 cultures.

In a few instances, callus formation was initiated within the anthers and burst through the wall as a mass approximately 3 mm in diameter (Fig. 4-4). This formation occurred in 4, 12, and 0 of the CH, FG, and FR anthers, respectively, and was observed only on Medium M, 14 and 20 d after culture initiation for FG and CH, respectively. A vegetative structure resembling a shoot formed from this callus in a culture of FG (Fig. 4-5) and CH (Fig. 4-6) within 34 d of culture initiation on Medium M (Table 4-3). Subsequently, the callus and structures ceased development, turned brown, and died within 6 d of transfer to medium supplemented with 0.01 mg BA L^{-1}.

In conclusion, varying flower bud sizes of the three peanut cultivars were found to correspond to different stages of microsporogenesis. Evidence indicates that the microspores of peanut respond differentially in culture.

Many of the microspores degenerated within 28 d of culture, while others remained uninucleate. Mitosis commenced by the 14th d of culture, with approximately 60% of the uninucleate microspores completing their first division by 28 d. No evidence of further embryo or pollen development was noted. Although plantlet development from anthers was not observed, root organogenesis occurred from the callus of somatic anther tissue of FG. Shoot structures emerged from callus that originated within an anther of both FG and CH, however, no further development occurred.

Table 4-1. Relationship between flower bud length and microsporogenesis of three peanut cultivars.

Microspore development stage	Flower bud length, mm†		
	Florigiant	Florunner	Chico
Tetrad	1.0	1.5	<1.0
Uninucleate	1.5-2.0	2.0-3.5	1.0-1.5
Binucleate	2.5-3.5	4.0-4.5	2.0-3.0
Mature pollen	4.0	5.0	3.5-4.0

† Bud length was determined by measuring from the base to the tip of the sepals.

Table 4-2. Microspore survival and development of Florigiant peanut over time.

Days In Culture‡	No. (%) microspore stage†			
	Degenerated	Uninucleate	Densely Stained Binucleate	Lightly Stained Binucleate
4	5(8)	55(92)		
7	15(25)	45(75)		
14	21(35)	27(45)	2(3)	10(17)
21	17(28)	29(48)	10(17)	4(7)
28	30(50)	12(20)	12(20)	6(10)
35	31(52)	11(18)	14(23)	4(7)
42	30(50)	13(22)	12(20)	5(8)
49	28(47)	14(23)	12(20)	6(10)
56	29(48)	12(20)	13(22)	6(10)
63	28(47)	13(22)	12(20)	7(12)
70	29(48)	12(20)	13(22)	6(10)

† Each value represents the mean response of 60 microspores from each of three anthers macerated individually in 1% acetocarmine.

‡ Anthers were cultured on agar solidified medium supplemented with 4 mg indole-3-acetic acid L^{-1} and 2 mg kinetin L^{-1}.

Table 4-3. In vitro morphological responses of excised anthers of three peanut cultivars.

Cultivar†	No.(%) of callusing anthers	No.(%) forming roots‡	No. forming shoots§
Chico (B)	10(23)*	0	0
Chico (M)	19(44)	0	1
Florunner (B)	8(19)	0	0
Florunner (M)	30(68)	0	0
Florigiant (B)	16(38)	5(15)	0
Florigiant (M)	41(93)	19(23)	1

* Mean no. of responding anthers of each cultivar differ for the M and B media at the 0.05 probability level based on t-tests.

† A total of 90 anthers of each cultivar were cultured for 70 days on media supplemented with either 4 mg indole-3-acetic acid L^{-1} and 2 mg kinetin L^{-1} (B) or 2 mg 1-naphthaleneacetic acid (NAA) L^{-1} and 0.5 mg 6-benzylaminopurine (BA) L^{-1} (M).

‡ Cultures that developed callus within 35 days of culture initiation were transferred to media supplemented with either 1 mg NAA L^{-1} and 2 mg BA L^{-1} (B) or 0.01 mg BA L^{-1} (M), respectively, to initiate organogenesis. Observations were taken 30 days after transfer.

§ Shoot structures developed on the M callus induction medium 34 days after culture initiation.

Figure 4-1. Uninucleate microspores of Florigiant peanut stained with acetocarmine after 7 days of culture.

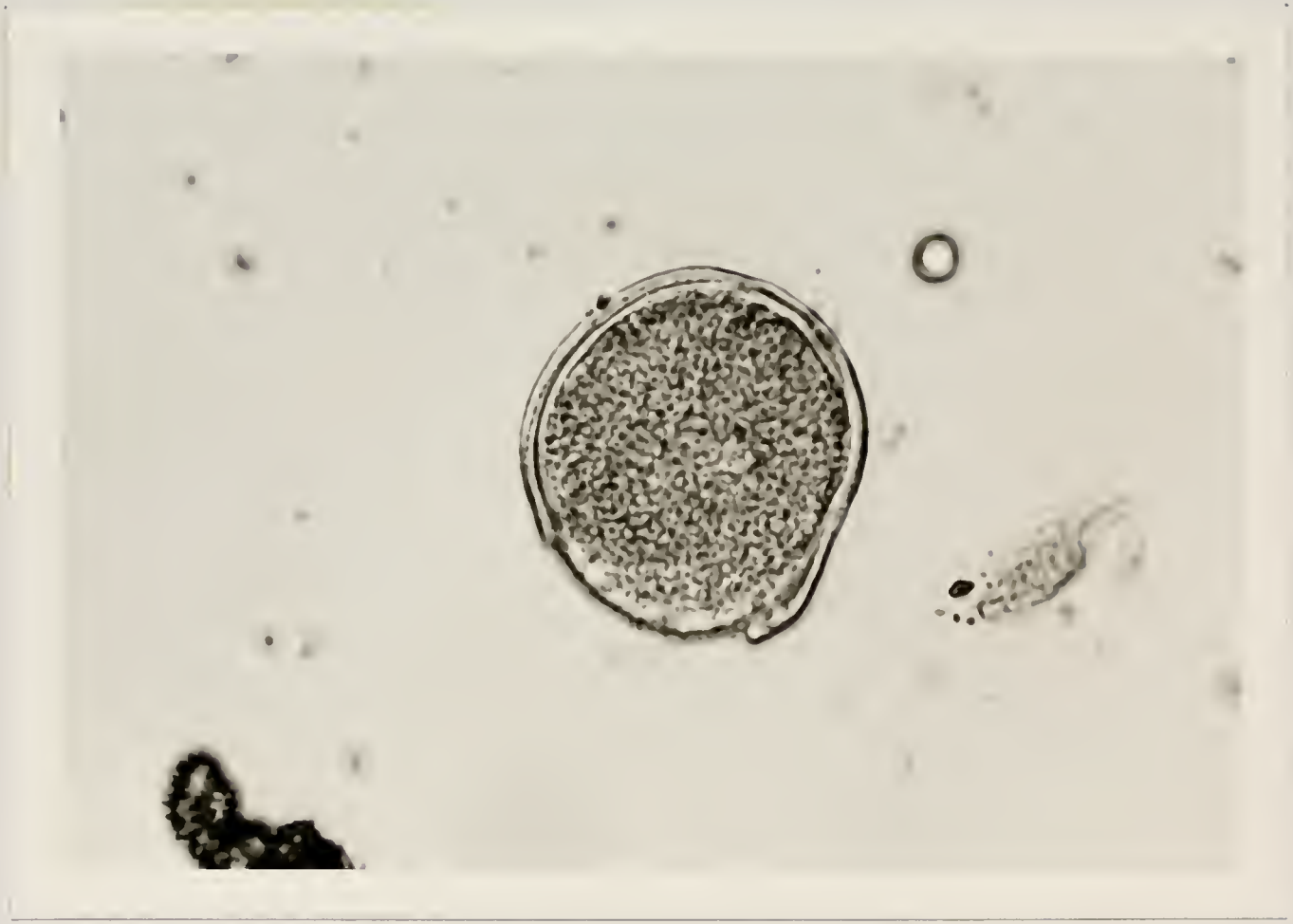

Figure 4-2. Densely stained binucleate microspore of Florigiant peanut after 28 days of culture.

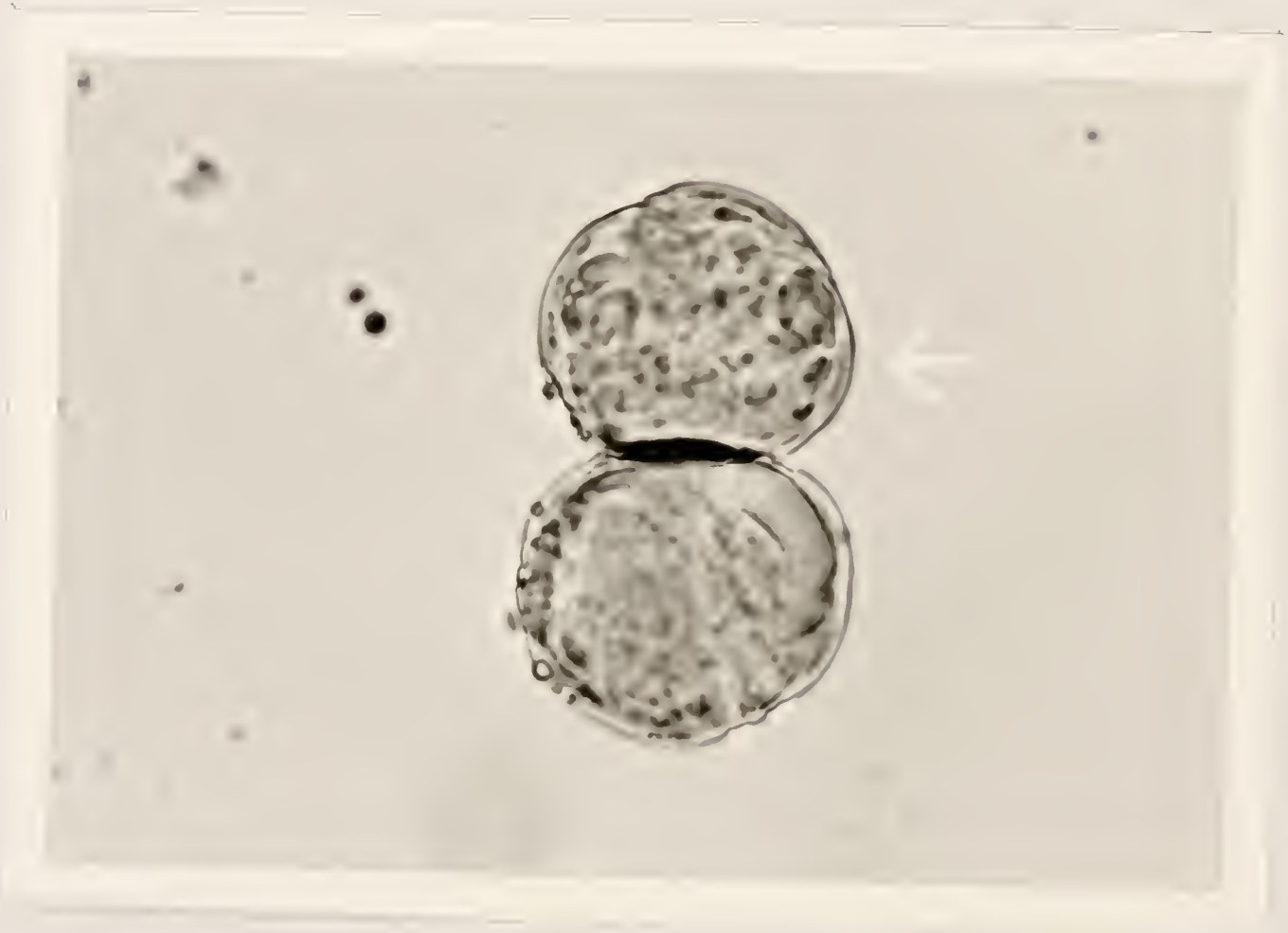

Figure 4-3. Lightly stained binucleate microspore of Florigiant peanut after 28 days of culture.

Figure 4-4. Callus tissue originating within an anther of Florigiant peanut and protruding through the wall after 14 days of culture.

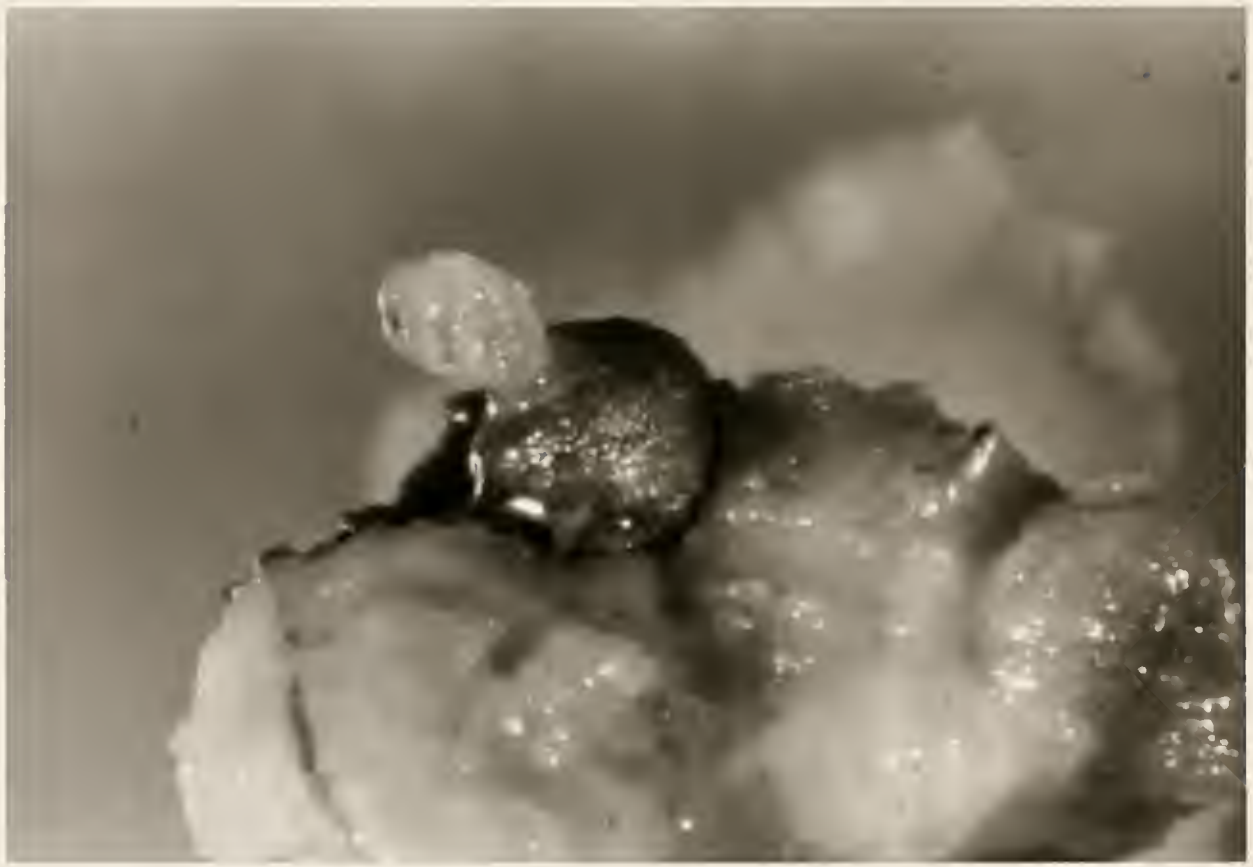

Figure 4-5. Shoot structure arising from callus initiated within an anther culture of Florigiant peanut.

Figure 4-6. Shoot structure arising from callus initiated within an anther culture of Chico peanut.

CHAPTER 5
SOMATIC EMBRYOGENESIS FROM IMMATURE COTYLEDON TISSUE OF PEANUT

Somatic embryogenesis is the initiation and development of embryos from somatic tissue (Ammirato, 1983). Haberlandt (1902) reported that somatic cells are totipotent and possess the ability to produce plants identical to the parent (Tisserat et al., 1979). He also predicted that this phenomenon would become apparent in tissue cultures of various plant species. A somatic embryo is the true manifestation of cell totipotency. It displays bipolar development with shoot and root produced as a structure which follows the developmental pathway of the zygotic embryo.

Somatic embryos develop in two ways (Sharp et al., 1980). The first way is direct embryogenesis where embryos arise directly from plant tissue. These embryos initiate from cells of explant material placed in culture conditions that induce such development (Tisserat et al., 1979). The second method is indirect embryogenesis where cell proliferation is a prerequisite for embryo formation (Sharp et al., 1980). The cells of the tissue differentiate to form callus, and are then induced toward embryo development (Ammirato, 1983).

Somatic embryogenesis was first recognized by Steward et al. (1958) and Reinert (1958) in tissue cultures of carrot. Since then, investigations have been widespread and a number of plant species have produced embryos in culture (Tisserat et al., 1979). Although a variety of monocots and dicots have produced somatic embryos in culture, attempts with large-seeded legumes have been unsuccessful until recently. Somatic embryo induction in soybean, Glycine max (L.) Merrill, (Barwale et al., 1986, Christianson et al., 1983, Gamborg et al., 1983, Kerns et al., 1985, Lippman and Lippman, 1984, Phillips and Collins, 1981), pea, Pisum sativum L., (Jacobsen and Kysely, 1984), and bean, Phaseolus vulgaris L., (Martins and Sondahl, 1984) were first reported in the 1980s. Further studies have revealed the direct and indirect production of somatic embryos from zygotic embryos of soybean (Lazzeri et al., 1985, Li et al., 1985, Ranch et al., 1985) and of broad bean, Vicia faba L. (Griga et al., 1987). The production of somatic embryos from the zygotic embryos of peanut, Arachis hypogaea L., has not yet been accomplished.

Griga et al. (1987) produced somatic embryos in callus and suspension cultures of broad bean. Callus was induced from immature seed cotyledon tissue cultured on medium containing 0.5 mg 2,4-dichlorophenoxyacetic acid (2,4-D) L^{-1}. Somatic embryos were then induced from this callus upon its transfer to both solid and suspension cultures of medium devoid of 2,4-D.

Lazzeri et al. (1985) regenerated whole plants from somatic embryos derived from immature zygotic embryos of soybean. Immature embryos were isolated from seed 4 mm long and cultured on media supplemented with either 5 mg 2,4-D L^{-1} or 10 mg 1-napthaleneacetic acid (NAA) L^{-1}. Somatic embryos developed on the cotyledon surfaces within 30 d, with higher frequencies of embryogenesis induced with 2,4-D than with NAA. However, embryos induced by 2,4-D had abnormal morphology, whereas those induced by NAA were normal. All embryos were removed from the cotyledon surfaces and transferred to medium containing 0.15 mg NAA L^{-1} and 0.03 mg L^{-1} each of 6-benzylaminopurine (BA), kinetin (KN), and zeatin (Z) to stimulate shoot development. The plantlets were then transferred to medium with 0.005 mg indole-3-butyric acid (IBA) L^{-1} to stimulate root development. As the plants developed root systems, they were transferred to pots containing autoclaved soil and grown to mature plants bearing seed.

Ranch et al. (1985) regenerated plants from the immature embryo-derived callus of 14 soybean genotypes. Whole embryos, embryo axes, and cotyledons were cultured on medium supplemented with 5 mg 2,4-D L^{-1}. In 3 wk somatic embryos developed with varying frequencies on both isolated cotyledons and cotyledons of whole embryos from all 14 genotypes. Embryo axes did not respond. Immature somatic embryos were removed from the cultures and transferred to medium containing 0.12 mg IBA L^{-1} and 0.1 mg gibberellic acid (GA) L^{-1} to

stimulate apical development. Within 30 d the mature embryos had developed into plants with three nodes.

Li et al. (1985) regenerated somatic embryos and plantlets from cold-treated immature soybean embryo fragments. The embryos were treated, cut into 2-mm segments, and then cultured on medium supplemented with 2 mg 2,4-D L^{-1} for 40 d. Callus formed from 85% of the fragments and was transferred to liquid medium containing 0.5 mg 2,4-D L^{-1} and 5% coconut milk, and was incubated on a rotary shaker at 100 rpm for 14 d. The cells were then filtered and transferred at 20 μL aliquots to petri plate covers to form hanging drop cultures for development into proembryos. These proembryos were transferred and maintained on solid medium containing 0.2 mg BA L^{-1} and 0.01 mg indole-3-acetic acid (IAA) L^{-1} for 40 d to induce development into globular and heart-shaped embryos. These were then returned to liquid medium supplemented with 0.2 mg BA L^{-1} and 0.01 mg IAA L^{-1} for differentiation into plantlets.

In summary, somatic embryos were derived from the isolated cotyledons, whole embryos, and fragmented embryo parts of immature soybean seed. The embryos developed from cotyledon tissue either directly or indirectly from callus. Auxins 2,4-D and NAA both were effective in embryo initiation. Although embryogenic cultures were derived from all genotypes tested, variation in the frequency of initiation among cultivars was noted.

This chapter investigated the somatic embryogenic response from the zygotic embryo cotyledons of three peanut genotypes cultured on media supplemented with a variety of growth regulator treatments.

Materials and Methods

Immature pods of three peanut cultivars, Florigiant (FG), Florunner (FR), and Chico (CH), were soaked in water containing 0.5% antimicrobial P-chloro-M-Xylenol for 2 h. Seeds were excised from the pods, and embryos ranging in size from 4 to 9 mm were isolated from the seed coats. The two cotyledons of each embryo were separated and the embryo axes removed. Cotyledons were surface sterilized in 70% ethyl alcohol for 5 min followed by three separate immersions in 2.6% sodium hypochlorite for 4 min each on a shaker. Cotyledons were rinsed three times with sterile deionized water. Cotyledons were placed into 100 X 15 mm plastic petri plates containing 25 mL of medium. The nutrient medium consisted of Murashige and Skoog salts (1962), 3% sucrose, 2 mg glycine L^{-1}, 0.5 mg nicotinic acid L^{-1}, 0.1 mg pyridoxine hydrochloride L^{-1}, and 0.8% Difco agar. Basal medium and a range of growth regulator treatments, 0.01, 0.1, 1, 5, and 10 mg L^{-1} of 2,4-D and NAA, and 10 mg BA L^{-1}, were prepared as treatments. The 12 media were adjusted to pH 5.8 with potassium hydroxide or hydrochloric acid and autoclaved at 1.1 kg cm^{-2} for 20 min. A total of 576 cotyledons of each cultivar, with four per petri plate, were cultured in a completely randomized design.

Cultures were maintained at 23°C with a 16 h daylength. General Electric F40CW•RS•WM fluorescent tubes were used which produced approximately 85 μmol m^{-2} s^{-1} when measured 215 mm from the tubes with a Li-190SB (LiCor) quantum sensor (400-700 nm range). After 10 d, the cultures with fungal or bacterial contamination were discarded. Cultures were transferred to fresh media of the same composition at 30-d intervals for 1 yr.

Results and Discussion

Callus formation occurred from only one of the 1,728 cotyledons cultured. A cotyledon of Chico cultured on medium supplemented with 5 mg 2,4-D L^{-1} developed callus 120 d after culture initiation. The callus originated within the explant and emerged through the abaxial surface. It continued to grow on subcultures to medium of the same composition and to medium supplemented with 5 mg BA L^{-1} and no auxin, but did not regenerate during the subsequent 245 d period. Comparatively, studies with soybean report direct development of somatic embryos, without callus formation, from zygotic embryo cotyledons within only 4 to 6 wk of culture initiation (Lazzeri et al., 1985, Ranch et al., 1985). There was no embryogenic response among the FG, FR, and remaining CH cotyledons cultured on the 12 media treatments for 1 yr. The majority of peanut cotyledons remained white during the entire culture period with 15, 12, and 8% of FG, FR, and CH, respectively, turning brown. Reports indicate that responsive cotyledons of soybean turn green within a

few days of culture initiation, and unresponsive cotyledons remain white or turn brown in culture (Lazzeri et al., 1985, Ranch et al., 1985).

The possibility of using this procedure for plant regeneration from immature cotyledon tissue of peanut will require further research to determine the influence of genotype. Ranch et al. (1985) evaluated 14 soybean genotypes and found that although there was variation in the frequency of somatic embryogenesis, all did produce embryos. The role of other factors such as growth regulator regime, tissue age, and environmental conditions also may require further evaluation.

CHAPTER 6
SUMMARY AND CONCLUSIONS

Success in peanut, Arachis hypogaea L., improvement programs through the use of in vitro techniques will require a knowledge about its capacity to regenerate and an understanding for its requirements in culture. In vitro morphogenesis occurred in a variety of tissue cultures from a number of peanut genotypes investigated in this study.

The regeneration response of explants taken from the seed of peanut provides an efficient system for successful high frequency in vitro regeneration of whole plants. Plant regeneration from cotyledon explants has been reported in peanut (Illingworth, 1968, 1974, Atreya et al., 1984, Bhatia et al., 1985) and also in other legumes (Oswald et al., 1977, Bharal and Rashid, 1979, Mehta and Mohan Ram, 1980, Mathews and Rao, 1984). Although shoot regeneration was achieved in these studies, rooting and establishment in soil often were difficult resulting in low success rates for whole plant development and survival. Little quantitative and comparative information on organ yields per explant type and the productivity of various genotypes were reported.

This technique of seed explant culture is the first simultaneous evaluation of the various explant types, and further provides a method for successful whole plant regeneration and establishment. Complete plants were regenerated

from in vitro-cultured embryo axes and embryonated and deembryonated cotyledons that were whole and sectioned. Optimal quantities of shoots were obtained with whole embryonated cotyledon explants cultured on solidified medium supplemented with 6-benzylaminopurine (BA). Excised shoots developed roots in vitro upon transfer to medium supplemented with 1 mg 1-napthaleneacetic acid (NAA) L^{-1}. Long-term prolific shoot cultures were produced and maintained using this procedure, and whole plants were continually produced from these cultures over a 34-wk period. This procedure was successful with 20 genotypes, although shoots were produced in quantities ranging from 4.6 to 14.4 per responding culture after 10 wk. Investigations of factors that influence the level of shoot productivity may be necessary on an individual basis to optimize this regeneration system for specific genotypes. In vitro-produced plantlets transferred to soil and placed in a greenhouse developed successfully, matured, and set seed. No phenotypic variants were observed among any of the plants produced in these experiments. Thus this system allows for the in vitro production of morphologically normal plants of peanut at high frequencies.

The regeneration response of leaf explants from the cultivated peanut and perennial peanut, Arachis glabrata Benth, was evaluated. Maximum induction of bud tissue from the cultivated peanut occurred on medium supplemented with 1 mg NAA L^{-1} and 5 mg BA L^{-1}. Shoot regeneration from immature leaflet tissue of peanut cultured on medium supplemented

with 1 mg NAA L^{-1} and 1 mg BA L^{-1} was previously reported by Mroginski et al. (1981) and Pittman et al. (1983). This present report indicates, however, that larger quantities of shoots can be induced with higher cytokinin concentrations. Increased frequency of shoot formation becomes imperative when applying this regeneration method to peanut improvement programs. Eighty-four percent of the bud tissue cultures from cultivated peanut continued growth and development into shoots within 120 d of transfer to medium supplemented with 5 mg BA L^{-1} and no NAA. These shoots developed roots in vitro within 30 d of transfer to auxin-supplemented medium. In vitro-produced plantlets were transferred to a greenhouse where they developed successfully. Although phenotypic variants were not observed among any of the regenerated plants, this system potentially offers a high degree of variability which can be exploited in peanut improvement. Bud regeneration was induced in 17 of the 20 genotypes tested. Genotypic differences in organogenic capacity require further investigation among peanut cultivars, because much variability was noted in the quantities of responding explants. Additional study of other explant sources may provide areas of increased organogenic activity for use in this tissue culture system.

The perennial peanut leaf explants callused and produced shoot meristems within 50 d of culture initiation. This is the first report of organogenesis in this species. Its pattern for organogenesis is indirect (Hicks 1980),

following a sequence of development including a callus stage. This indirect organogenesis pattern is primary explant → callus → meristem → organ. The largest number of organogenic calli occurred on media supplemented with either 3 or 5 mg BA L^{-1}. The majority of shoot meristems initially produced did not develop and differentiate to become whole plants. As with culture initiation and optimum shoot meristem production, fine-tuning of this tissue culture system may allow greater numbers of meristems to follow the normal course of development and germination. This in vitro regeneration system would allow for the potential mass production of plants from one exhibiting or carrying traits desirable for improved lines. It also provides an alternative clonal propagation method for crops, such as perennial peanut, that are not normally seed propagated.

The induction of microspore-derived embryos and the production of haploid plants from the cultivated peanut were evaluated, and limited success was achieved. Microspore survival and development was investigated and provided evidence that uninucleate microspores respond differently in culture. Many of the microspores degenerated within 28 d of culture, while others remained uninucleate. Mitosis occurred by the 14th d of culture, with approximately 60% of the uninucleate microspores completing their first division by 28 d. Interestingly, 10% of these binucleate microspores were clearly segmented with two nuclei of similar size. This phenomenon has been reported in tobacco (Sunderland and

Wicks, 1969), with additional microspore cell division resulting in complete embryo development. Although there was no evidence of further embryo or pollen development from the binucleate microspores of peanut during the 70-d observation period, additional time may have been required for embryogenesis. Also, there may have been inhibitors to embryogenesis released from anther tissue or degenerated microspores. Future research should concentrate on the development of a system to obtain a pure culture of these binucleate microspores. This will provide isolation from unresponsive and degenerated microspores, allowing competent microspores to continue development along the embryogenic pathway.

Structures resembling embryos emerged from callus that was initiated within the walls of two anthers, but no further development of these structures occurred. Root organogenesis occurred from somatic anther tissue-derived callus, however, shoot development did not occur. The formation of roots provides evidence for morphogenesis and possible whole plant regeneration, which would provide an additional source of genetic diversity for this important crop.

The formation of somatic embryos and callus from the cotyledons of immature zygotic embryos was investigated in three peanut cultivars cultured on media supplemented with a variety of growth regulator treatments. Only one of the 1,728 cotyledons cultured developed callus. The possibility of utilizing this procedure for plant regeneration will

require much research in the future to determine the influence of genotype. The role of other factors such as growth regulator regime, tissue age, and environmental conditions will also require further evaluation.

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BIOGRAPHICAL SKETCH

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Alexandra received a Bachelor of Science degree in horticulture in 1979 from the Pennsylvania State University, University Park, Pennsylvania. She attended the University of Florida, and obtained a Master of Science degree in agronomy in 1981. In 1984, Alexandra began study towards a Doctor of Philosophy degree to be awarded in December 1988.

I certify that I have read this study and that in m opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Frank P. Gardner, Chair
Professor of Agronomy

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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